Enzymatic Synthesis of Oligosaccharides from Maltose by Germinated Green Gram (Phaseolus radiatus)

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A rat liver enzyme, capable of synthesizing linear dextrins from maltose, was discovered in this laboratory (1). Later, a similar enzyme was identified and highly purified from bovine plasma by Miller and Copeland (2, 3). The mechanism of rat liver transglycosylase has recently been studied by Stetten (4). Transglycosylases which are capable of oligosaccharide synthesis from disaccharides have been reported in molds (5) and in Escherichia coli (6). The E. coli and liver enzyme resemble each other in giving the same linear dextrins when acting on maltose whereas enzymes from molds generally give branched chain oligosaccharides (panose, isomaltose, isomaltotriose).

Among plants transfructosidation has been reported extensively (5). Reports on transglycosidation resulting in the synthesis of oligosaccharides from disaccharides are lacking. Only Duncan et al. (7) have briefly reported the presence of an enzyme in sea weeds which synthesizes malto-oligosaccharides.

In this paper we describe the presence of an enzyme in green gram similar to the transglucosidase of rat liver. The enzyme is absent in the resting seeds and develops only during their germination.

EXPERIMENTAL

Substrates—Maltose was obtained from Nutritional Biochemicals Corporation. This preparation contained small amounts of glucose and traces of maltotriose. For the isolation of oligosaccharides it was purified by adsorption on acid-washed charcoal column (British Drug Houses, Ltd.-activated charcoal) and elution with 7.5% aqueous ethanol. The resulting sugar solution was free of maltotriose.

Crystalline glucose 1-phosphate was kindly prepared for us by Dr. T. Ramasarma of this laboratory according to Hanes (8). Maltotriose and maltotetraose were prepared by the partial hydrolysis of amylose and were separated on a charcoal column as described by Whelan et al. (9).

Isolation of Oligosaccharides Formed by Action of Green Gram Transglucosidase—Twenty grams of 4-day-old green gram seedlings were ground with cold distilled water and sterile sand in a mortar. The suspension was centrifuged and the supernatant fluid was dialyzed against distilled water at 0–5°. After 24 hours of dialysis the enzyme solution was centrifuged and the supernatant solution was fractionated with ethanol. To the extract (100 ml) at 0° was slowly added an equal volume of ethanol at −5° with constant stirring. The precipitate obtained was centrifuged out at 0° and to the supernatant fluid more cold ethanol was added to bring its concentration to 80%. The precipitated protein was collected, washed with chilled acetone, and dried over P₂O₅ at 0°. It was obtained as a dull white powder, soluble in water, and was used for the time course study of the green gram transglucosidase.

Analytical Methods—Circular paper chromatography as described by Giri and Nigam (12) was used in the present investigation. The solvents for the separation of sugars were butanol-acetone-water (7:2:1), butanol-acetic acid-water (4:1:5) and butanol-pyridine-water (6:4:3). The general reagent, aniline diphenylamine phosphate (12) was used for spraying the chromatograms and for locating the sugar bands. Elution of the sugars was carried out by eluting sugars from the paper strips cut out from a well developed chromatogram at the expected positions and by subsequently determining reducing power with Nelson's arsenomolybdate colorimetric reagent (13). Reference curves were prepared for glucose, maltose, maltotriose, and maltotetraose under the same conditions, with known amounts of the sugars. Filter paper strips of equal areas lacking sugar provided the controls.

RESULTS

Preparation of Green Gram Transglucosidase—Green gram seeds were obtained locally and were allowed to germinate in porcelain troughs under a thin layer of water. The seeds germinated during a period of 24 hours but were allowed to grow till the seedlings reached a length of 2 to 3 inches. The whole plants were then ground with cold distilled water and sterile sand in a mortar. The suspension was centrifuged and the supernatant fluid was dialyzed against distilled water at 0–5°. After 24 hours of dialysis the enzyme solution was centrifuged and the supernatant solution was fractionated with ethanol. To the extract (100 ml) at 0° was slowly added an equal volume of ethanol at −5° with constant stirring. The precipitate obtained was centrifuged out at 0° and to the supernatant fluid more cold ethanol was added to bring its concentration to 80%. The precipitated protein was collected, washed with chilled acetone, and dried over P₂O₅ at 0°. It was obtained as a dull white powder, soluble in water, and was used for the time course study of the green gram transglucosidase.

Isolation of Oligosaccharides Formed by Action of Green Gram Transglucosidase on Maltose—Twenty grams of 4-day-old green gram seedlings were ground with 50 ml of cold distilled water. The suspension was centrifuged, dialyzed for 24 hours in the cold, and used as an enzyme source for the formation of oligosaccharides. A solution of purified maltose containing 10 g of the sugar in 25 ml of 0.01 M phosphate buffer, pH 6.8, was incubated at room temperature with 25 ml of the dialyzed enzyme solution for 4 days under a layer of toluene. After the incubation the digest was heated in a water bath for 3 minutes to inactivate the enzyme and precipitate part of the protein. The solution was centrifuged and the clear supernatant was introduced at the top of a charcoal Celite column (4.8 X 54 cm). The charcoal column was then developed with various concentrations.
butanol-pyridine-water (6:4:3), 8 hours; 2. butanol-acetone-water layer.

- 948 Oligosaccharide Synthesis from Maltose solutions containing I and II were concentrated separately under elution by 20% ethanol to elute the tetrasaccharide (II). The reduced pressure left I (190 mg) and II (85 mg) in light yellow and the methanol solution on concentration to dryness under reduced pressure. The syrups were extracted with methanol flakes.

- 948 To elute the trisaccharide (I) followed later after its complete chromatography) the ethanol concentration was raised to 15% containing glucose and maltose were rejected. After the complete removal of maltose was assured (as observed by paper chromatography) the ethanol eluates of aqueous ethanol as described by Whelan et al. (9) for the separation of maltodextrins. The water and 7.5% ethanol eluates were used for determining Rf values which corresponds in position to oligosaccharides I and II in three solvents were the same as obtained for maltotriose and maltotetraose (Table I). A plot of \( \log \left( \frac{1}{R_f} - 1 \right) \) against the number of hexose units per molecule shows that glucose, maltose, oligosaccharides I and II give a straight line (Fig. 1). A similar straight line would be obtained if I and II are substituted by maltotriose and maltotetraose.

- 948 The color reaction of I and II on paper after spraying with aniline diphenylamine phosphate was blue and matched exactly with that given by maltose, maltotriose, and its homologues and distinctly different from isomaltose or isomaltotriose which give brown-colored bands. Molecular weight determination with the hypoiodite method of Hirst et al. (14) showed that oligosaccharides I and II had chain lengths of 3 and 4 glucose units.

Additional data for the support of I as maltotriose and II as maltotetraose is given by their rotation and the rotation of their acetates. I gave \( [\alpha]_D^{20} +161^\circ (c = 4.5 \text{ in } H_2O) \) maltotriose \( [\alpha]_D^{20} +100^\circ (15). \) II gave \( [\alpha]_D^{20} +176.4^\circ (c = 23 \text{ in } H_2O) \) maltotetraose \( [\alpha]_D^{20} +165.5^\circ (15). \)

Preparation of Acetates—Fifty milligrams of each of the oligosaccharides were suspended in 1 ml of acetic anhydride and 1 ml of pyridine. The mixture was left at room temperature for 48 hours during which time the sugars went completely in solution. The solution was then added to 50 ml of ice-cold water. Twenty-four hours later, the precipitate was collected, washed with an excess of cold water and 25% cold ethanol, and then dried over P_2O_5. The yield for oligosaccharide I acetate was 70 mg and for oligosaccharide II acetate 75 mg. Efforts at crystallization with 50% ethanol were not successful. The optical rotation for oligosaccharide I acetate was \( [\alpha]_D^{20} +104.8^\circ (\text{acetyl content } = 49.1\%) \) and for maltotriose acetate \( [\alpha]_D^{20} +106.5^\circ (\text{acetyl content } = 49.0\%) \) (6). The optical rotation for oligosaccharide II acetate was \( [\alpha]_D^{20} +127.2^\circ (\text{acetyl content } = 47.7\%) \); and for maltotetraose acetate is \( [\alpha]_D^{20} +127.7^\circ (\text{acetyl content } = 48.0\%) \) (6).

Identification of Oligosaccharides I and II—Both I and II on complete hydrolysis with acid gave glucose as the only product, which was identified by paper chromatography in butanol-acetone-water and in butanol-pyridine-water systems. I (10 mg) on partial hydrolysis with 0.1 N sulfuric acid for 1 hour and subsequent neutralization with BaCO_3 gave 3 bands corresponding to glucose, maltose, and maltotriose when subjected to paper chromatography. On similar treatment II (10 mg) gave 4 bands which moved the same distance as covered by glucose, maltose, maltotriose, and maltotetraose. The absence of any band corresponding to isomaltose was indicative of the absence of 1-6 linkage in the oligosaccharides. The Rf values for the oligosaccharides I and II in three solvents were the same as obtained for maltotriose and maltotetraose (Table I).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Maltotriose</th>
<th>Oligosaccharide I</th>
<th>Maltotetraose</th>
<th>Oligosaccharide II</th>
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<td>0.21</td>
<td>0.21</td>
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<tr>
<td>2</td>
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<td>0.15</td>
<td>0.15</td>
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<td>0.17</td>
<td>0.16</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

A control experiment was carried out by incubation of a similar dialyzed extract of 4-day-old green gram seedlings with distilled water replacing maltose solution. Chromatography at zero time and after 96 hours of incubation under a layer of toluene did not reveal any sugar-reacting spots on the chromatogram after spraying with aniline diphenylamine phosphate. Hence the isolated oligosaccharides were neither present in the initial enzyme extract nor formed in the absence of maltose, from endogenous substrate present in the enzyme extract. Also no change in the formation of the oligosaccharides took place when phosphate buffer was replaced by water solution of maltose. The formation of the oligosaccharides can therefore be regarded as nonphosphorolytic.

Identification of Oligosaccharides I and II—Both I and II on...
II located by spraying an adjacent sector with aniline diphenylamine phosphate.

**Time Course of Transglucosidase Reaction**—Twenty milligrams of the enzyme powder were weighed accurately into clean test tubes. The reaction was started by the addition of 1 ml of a buffered solution of 20% maltose in 0.02 M phosphate buffer, pH 6.8. The tubes were shaken to dissolve the enzyme and incubated at 37° under a layer of toluene. The reaction in each tube was stopped at a different interval of time by heating the tube in a boiling water bath for 3 minutes. The digest was centrifuged and the supernatant fluid was analyzed chromatographically. Control digests which were boiled just after the addition of the maltose solution to the enzyme showed no oligosaccharide formation. A typical chromatogram showing the synthesis of oligosaccharides at different intervals of time is shown in Fig. 2. It is observed that the enzyme forms of trisaccharides in a period of 12 hours followed by the appearance of a tetrasaccharide at later intervals. This would suggest that the trisaccharide is the acceptor of an additional glucose residue which results in the formation of the tetrasaccharide. No oligosaccharide larger than the tetrasaccharide was observed even after long periods of incubation (6 days). Also complete disappearance of maltose did not take place. Fig. 3 shows the progress of maltose hydrolysis to glucose and the subsequent formation of maltotriose and maltotetraose.

**DISCUSSION**

Although transglucosidases have been encountered extensively in molds and bacteria, instances of their occurrence in the plants are rare. Perhaps, it is because the seeds elaborate this enzyme only at a certain stage of development. This view is supported by our observation that the seeds were totally inactive and the maximum transglucosidase activity occurred during the early period of germination. The enzyme was purified by fractional precipitation with ethanol; it converted maltose to glucose, maltotriose, and maltotetraose. The last two sugars were isolated from a large scale digest by charcoal column chromatography and identified as maltotriose and maltotetraose by migration on paper, total and partial acid hydrolysis, optical rotation, hypoiodite oxidation, and by the preparation of acetyl derivatives. Both sugars also gave positive tests when examined for their priming activity in enzymic amylose synthesis.

The time activity relationship shows that synthesis occurs concurrently with the hydrolysis of the substrate. Also maltotriose is formed earlier than maltotetraose. The mechanism for transglucosidases of green gram can be explained by an interpretation similar to that suggested for other transglucosidases (5). Thus a glucose moiety from maltose would be transferred to maltose molecule resulting in the formation of maltotriose. The maltotriose formed in turn accepts another glucose moiety, probably from maltose, to synthesize maltotetraose. It is interesting to note that both rat liver and green gram transglucosidases bring about the synthesis of straight chain oligosaccharides with 1:4 glucosidic linkages whereas the mold transglucosidases form oligosaccharides with 1:6 linkages.

**SUMMARY**

Green gram seedlings are found to contain an enzyme capable of oligosaccharide synthesis from maltose. The two higher saccharides formed were isolated and characterized as maltotriose and maltotetraose by a number of methods. The green gram transglucosidase was purified by fractional precipitation with ethanol and the time activity relationship was determined.

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